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IN VITRO PROPAGATION OF EIGHT SPECIES OR SUBSPECIES OF *TURBINICARPUS* (*CACTACEAE*)

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Summary

In vitro propagation systems by means of areole activation were developed for *Turbinicarpus laui*, *T. lophophoroides*, *T. pseudopectinatus*, *T. schmiedickeanus* subsp. *flaviflorus*, *T. schmiedickeanus* subsp. *klinkerianus*, *T. schmiedickeanus* subsp. *schmiedickeanus*, *T. subterraneus*, and *T. valdezianus*. *In vitro*-germinated seedlings were used as a primary source of explants. Multiple shoot formation from areoles was achieved for three explant types (apical, lateral, and transverse), cultured on Murashige and Skoog (MS) basal medium supplemented with 3% sucrose, 10 g l^{-1} agar and several treatments with cytokinins. Efficiencies were in the range from 7.8 shoots per explant in *T. valdezianus* up to 19.7 shoots per explant in *T. pseudopectinatus*, using the best treatment for each species and in a single proliferation cycle. Four of the studied species responded best when 6-benzylaminopurine (3.3–8.8 μM) was used, while 6-(γ,γ-dimethylallylamino)purine (19.7–24.6 μM) showed better results in two species. The two remaining species showed no significant differences in their response to both cytokinins. Regarding explant type, the best results were obtained with transverse cuts for five species, with apical explants for one species, and the two remaining species showed no significant differences among the explants tested. Rooting of the *in vitro*-generated shoots was achieved most efficiently on half- or full-strength MS basal medium. Rooting frequencies were in the range from 54.2 to 94.2%, and the frequency of survival of the plants once transferred to soil was 91.6% on average.

Key words: areole activation; cytokinin; micropropagation; tissue culture.

Introduction

The genus *Turbinicarpus* includes 24 species and several subspecies of small and globose cacti which inhabit areas with limestone or gypsum rocks throughout northern Mexico, from Coahuila south into Guanajuato. Most species have limited ranges, often restricted to one or a few hills. These cacti are popular among collectors due to their high ornamental value and small size, which makes these plants easy to maintain. Unfortunately, the desirability of these cacti has resulted in depredation of many populations by illegal collecting and now the entire genus is included in Appendix I of CITES (Glass, 1998; Anderson, 2001). In Mexico, all the species of *Turbinicarpus* are officially protected by the Norma Oficial Mexicana (NOM-059-ECOL-2001); however, this has not stopped the pillage of the wild populations nor the destruction of their habitat.

On the other hand, because *Turbinicarpus* plants are self-sterile and produce few offsets, propagation by seeds is not completely satisfactory. For this reason, a desirable action in order to safeguard these species is to improve or develop new and efficient propagation techniques. *In vitro* culture techniques have been developed into a successful means of propagating several cacti asexually. These techniques have the potential to produce many plants in a short time and in minimal space (Hubstenberger et al., 1992). Some threatened species have been propagated with success using *in vitro* culture techniques. Examples of this are *Mammillaria sanangelensis* (Martínez-Vázquez and Rubluo, 1989), *Aztekium ritteri* (Rodríguez-Garay and Rubluo, 1992), *Pelecyphora aselliformis* and *P. strobiliformis* (Pérez-Molphe-Balch and Dávila-Figueroa, 2002), and *Ariocarpus kotschoubeyanus* (Moebius-Goldammer et al., 2003). With regard to *Turbinicarpus*, Mata-Rosas et al. (2001) reported the production of adventitious shoots from callus obtained from longitudinal sections of *in vitro*-germinated seedlings of *T. laui*. In the present study, we describe the production of multiple shoots by means of areole activation as well as their rooting and soil establishment for eight threatened and endemic species or subspecies of *Turbinicarpus*.

Materials and Methods

Plant material. *In vitro*-germinated seedlings of *Turbinicarpus laui* Glass & R. Foster, *T. lophophoroides* (Werdermann) Buxbaum, *T. pseudopectinatus* (Backeberg) Glass & R. Foster, *T. schmiedickeanus* subsp. *flaviflorus* (G. Frank & A. B. Lau) Glass, *T. schmiedickeanus* subsp. *klinkerianus* (Backeberg & H. Jacobsen) N. P. Taylor, *T. schmiedickeanus* subsp. *schmiedickeanus* (Boedeker) Buxbaum & Backeberg, *T. subterraneus* (Backeberg) A. D. Zimmerman and *T. valdezianus* (H. Moeller) Glass & R. Foster were used as explant sources. To establish the *in vitro* cultures, seeds were washed five times with 0.1% Extran (Merck-México, Naucalpan de Juárez, Mexico) in water, then disinfected for 1 min in 70% ethanol, 25 min in 2% sodium hypochlorite, and rinsed four times under aseptic conditions with sterile distilled water. Seeds were germinated in culture vessels containing MS medium (Murashige and Skoog, 1962) with 30 g l^{-1} sucrose and solidified with 10 g l^{-1} agar (Sigma-Aldrich, St. Louis, MO, USA). All culture media were adjusted to pH 5.7 with NaOH. The culture

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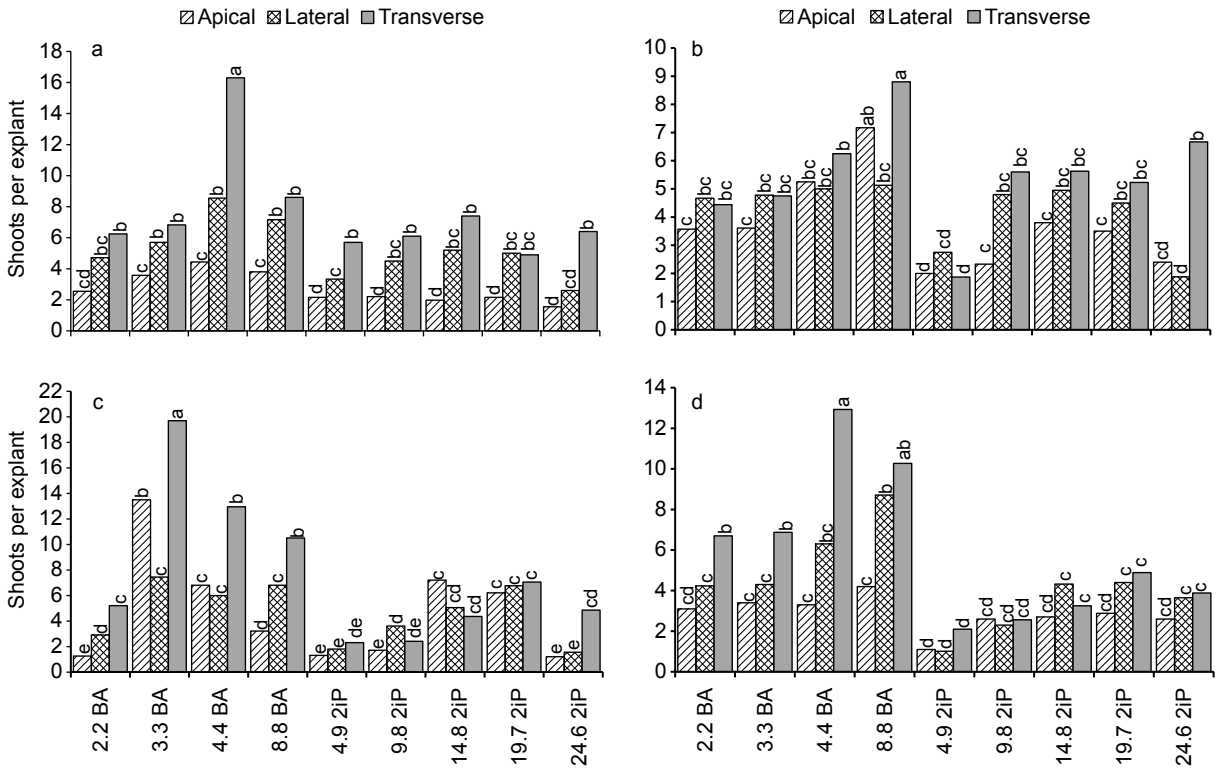

FIG. 1. Shoot production by areole activation in apical, lateral, and transverse explants of *Turbinicarpus*. *a*, *T. laui*; *b*, *T. lophophoroides*; *c*, *T. pseudopectinatus*; *d*, *T. subterraneus*. Values followed by the same *letters* do not significantly differ at $P \leq 0.05$, using the Tukey–Kramer multiple range test. Combined results are shown for two or three independent experiments.

jars with 30 ml of medium each were sterilized in an autoclave at 121°C for 20 min. Five seeds were planted per culture jar. The cultures were maintained for 4–5 mo. under continuous fluorescent light (54 μmol $m^{-2} s^{-1}$, daylight lamps) at 25 ± 2°C. These same basal medium and incubation conditions were used in all subsequent experiments. Due to the difficulty of obtaining plant material from *Turbinicarpus*, the plantlets obtained from the seeds were subjected to a preliminary *in vitro* multiplication cycle with the purpose of obtaining enough plant material for the following experiments. For this, roots were separated and the plantlets were inoculated vertically onto MS basal medium with 2.22 μM 6-benzylaminopurine (BA) with the purpose of producing new shoots from the areoles. These shoots were collected and transferred to basal medium with 3 $g l^{-1}$ activated charcoal (Phytotechnology Laboratories, Shawnee Mission, KS, USA) for growth. From these preliminary trials sufficient plant material was obtained for subsequent experiments.

Areole activation. Shoots (10–15 mm) obtained from the preliminary *in vitro* multiplication cycle were used as a source of explants for the mass proliferation experiments. This proliferation was carried out by means of axillary bud activation. Three explant types were tested: apical explants, lateral explants (shoots without apex cut longitudinally), and transverse segments approximately 4 mm wide (shoots without apex cut transversely). The explants were placed into 473-ml polypropylene culture vessels (Phytotechnology Laboratories) containing 80 ml of MS basal medium with a cytokinin. Treatments with 6-benzylaminopurine (BA; 2.2, 3.3, 4.4, 8.8 μM) or 6-(γ,γ-dimethylallylamino)purine (2iP; 4.9, 9.8, 14.8, 19.7, 24.6 μM) were tested with the purpose of selecting the most efficient for the generation of buds through areole activation. We used 20 explants of each type per treatment. The number of shoots produced in each explant was recorded after 45–50 d of incubation. These experiments were conducted two or three independent times for each species. A completely random experimental design was used. Data were analyzed through ANOVA and means were compared by the Tukey–Kramer multiple range test at the 5% level.

Rooting of shoots. Shoots were collected from the induction medium for rooting experiments. The rooting technique consisted of transferring 10–20-mm shoots to: (1) half-strength MS basal medium; (2) full-strength MS basal medium; (3) full-strength MS basal medium with 3 $g l^{-1}$ activated charcoal; and (4) full-strength MS basal medium with 4.90 μM indole-3-butyric acid (IBA). For this rooting experiment 50 shoots of each species were used per treatment. The production of roots was evaluated 6 wk after initiating the experiment. Shoots that developed at least three roots ≥ 15 mm in length were scored positively. The experiments were conducted two independent times for each species.

Acclimatization and transfer to soil. Rooted plants were transplanted to pots containing a mix of ground sand and commercial potting soil (1:1), covered with plastic bags for 2–3 wk to prevent desiccation and to allow acclimatization, and then transferred to the greenhouse. Survival percentages were determined 16 wk after transplantation.

RESULTS AND DISCUSSION

Germination occurred gradually starting 14 d after the inoculation of seeds. Germination frequencies registered at the third month were from 46% in *Turbinicarpus valdezianus* to 90% in *T. subterraneus*. The preliminary *in vitro* multiplication cycle gave satisfactory results in all the species since it allowed for increasing the initial plant material to provide sufficient explants to test several treatments with cytokinins. In previous experiments with the same species we have observed that these growth regulators are indispensable for the generation of shoots through areole activation. Our control treatments without cytokinins were unable to induce shoot proliferation (data not shown). On the contrary, when cytokinins were included in the culture media, shoots were obtained in all the species studied and with all tested treatments (Figs. 1 and 2). However, differences were found in the number of shoots per

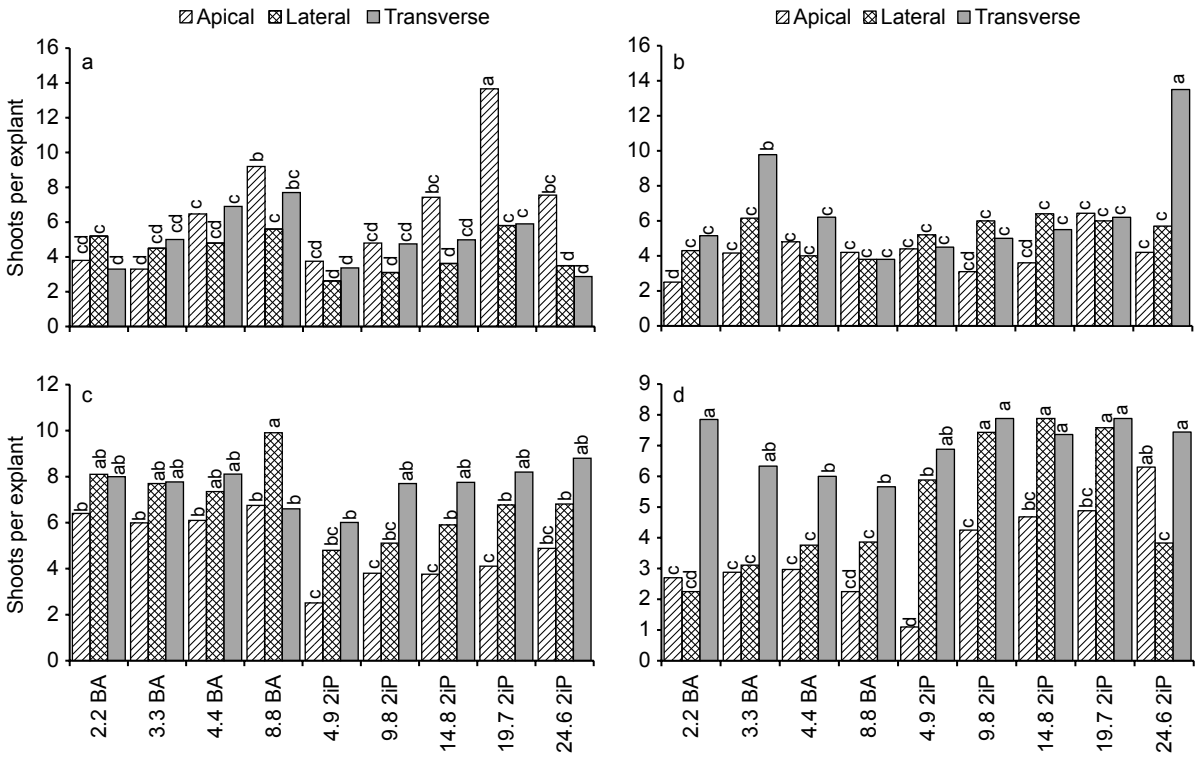

FIG. 2. Shoot production by areole activation in apical, lateral, and transverse explants of *Turbinicarpus*. *a*, *T. schmiedickeanus* subsp. *flaviflorus*; *b*, *T. schmiedickeanus* subsp. *klinkerianus*; *c*, *T. schmiedickeanus* subsp. *schmiedickeanus*; *d*, *T. valdezianus*. Values followed by the same *letters* do not significantly differ at $P \leq 0.05$, using the Tukey–Kramer multiple range test. Combined results are shown for two or three independent experiments.

explant generated amongst the tested species. In all cases, shoots emerged from the areoles of inoculated explants (Fig. 3*a*–*d*). Regarding the number of shoots obtained per explant, the range spanned from 7.8 in *T. valdezianus* to 19.7 in *T. pseudopectinatus*, considering the best treatment for each species and in a single proliferation cycle.

These efficiencies are similar to or greater than the ones reported for species in other cacti genera also using areole activation as a means of proliferation. Pérez-Molphe-Balch et al. (1998) report a range from 2.1 to 17.5 shoots per explant in a study conducted on 21 species in 10 genera of *Cactaceae*. Elias-Rocha et al. (1998) report seven shoots per explant for *Mammillaria sphacelata*. There are also reports of 13.7 and 12.3 shoots per explant in *Pelecyphora aselliformis* and *P. strobiliformis*, respectively (Pérez-Molphe-Balch and Dávila-Figueroa, 2002) and 5.3, 3.8, and 4.3 shoots per explant in *Carnegiea gigantea*, *Pachycereus pringlei*, and *Stenocereus thurberi*, respectively (Pérez-Molphe-Balch et al., 2002). The use of the cytokinins BA and 2iP in cacti micropropagation has been reported previously (Escobar et al., 1986; Hubstenberger et al., 1992). In the species *Turbinicarpus laui*, *T. lophophoroides*, *T. pseudopectinatus*, and *T. subterraneus*, the best response in number of shoots per explant was obtained using BA, whilst *T. schmiedickeanus* subsp. *flaviflorus* and *T. schmiedickeanus* subsp. *klinkerianus* generated a greater number of shoots in response to 2iP. This was confirmed by statistical analysis. However, in the species *T. schmiedickeanus* subsp. *schmiedickeanus* and *T. valdezianus*, no significant differences were found between the responses to these cytokinins (Figs. 1 and 2). This confirms the fact that each cacti species, even within the same genus, responds differently to growth regulators, hence *in vitro* proliferation systems must be developed for each one of them specifically (Hubstenberger et al., 1992).

Regarding explant type, most species showed a greater response when using transverse explants (Fig. 3*a*, *c*, *d*). Only the species *T. schmiedickeanus* subsp. *flaviflorus* responded best when using apical explants, whilst in *T. schmiedickeanus* subsp. *schmiedickeanus* and *T. valdezianus* the explant type did not influence the response. In the developed micropropagation systems, the number of shoots that can be generated from an explant at the end of a proliferation cycle is lower than the one reported by Mata-Rosas et al. (2001), who proposed a regeneration system from callus for *T. laui*, obtaining up to 269 shoots per original explant after induction with 8.8 μM BA and 3 mo. of incubation. However, the production of new plants through a callus phase can generate somaclonal variation, which is undesirable when it is intended to conserve the intact natural cactus germplasm (Oliveira et al., 1995). We chose to develop proliferation systems based on areole activation, because plants generated directly from meristematic structures are considered to be more genetically stable (Machado and Prioli, 1996). In fact, in several of the tested treatments, especially those containing BA, the emergence of callus was observed in the wounded surface of the explants (Fig. 3*a*–*d*). In most cases this tissue was not abundant, but in some instances its growth was greater and adventitious shoots were produced after

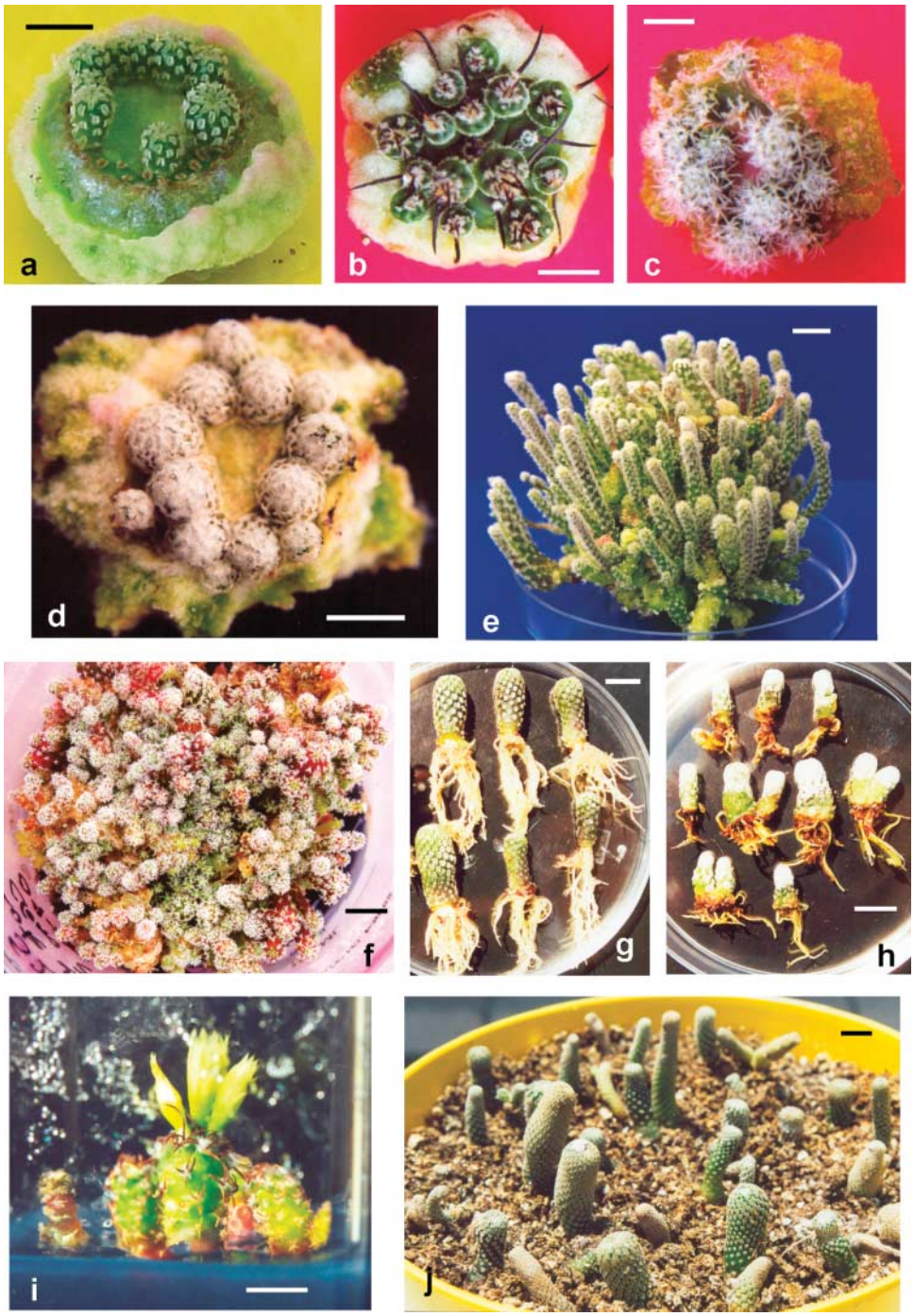

FIG. 3. *In vitro* propagation of *Turbinicarpus*. *a*, Shoot proliferation in a transverse explant of *T. pseudopectinatus* obtained with 3.3 μ*M* BA after 45 d of culture initiation. *b*, Shoot proliferation in an apical explant of *T. schmiedickeanus* subsp. *klinkerianus* obtained with 14.8 μ*M* 2iP after 40 d of culture initiation. *c*, Shoot proliferation in a transverse explant of *T. laui* obtained with 4.4 μ*M* BA after 45 d of culture initiation. *d*, Shoot proliferation in a transverse explant of *T. valdezianus* obtained with 2.2 μ*M* BA after 50 d of culture initiation. *e*, Secondary shoot proliferation in a *T. subterraneus* explant obtained with 4.4 μ*M* BA after 120 d of culture initiation. *f*, Secondary shoot proliferation in a *T. lophophoroides* explant obtained with 8.8 μ*M* BA after 100 d of culture initiation. *g*, Shoots of *T. pseudopectinatus* rooted in 100% basal medium. *h*, Shoots of *T. valdezianus* rooted in 50% basal medium. *i*, Shoots of *T. schmiedickeanus* subsp. *flaviflorus* cultured in 100% basal medium with 3 g l^{-1} activated charcoal, showing the appearance of flowers. *j*, *In vitro*-generated plants of *T. pseudopectinatus* growing in soil. *Bars* = 10 mm.

TABLE 1

ROOTING AND ACCLIMATIZATION OF *IN VITRO*-GENERATED SHOOTS OF EIGHT SPECIES OR SUBSPECIES OF *TURBINICARPUS*

Species	*In vitro* rooting treatment	Rooting frequency (%)	Survival *ex vitro* (%)
T. laui	1/2MS	82.0 ± 4.8	87.8 ± 3.5
	Full MS	89.7 ± 2.2	71.4 ± 7.8
	Full MS + 3 g l^{-1} AC	75.6 ± 3.3	77.6 ± 8.1
	Full MS + 4.9 μ*M* IBA	76.2 ± 5.8	64.7 ± 13.5
T. lophophoroides	1/2MS	90.8 ± 3.8	77.9 ± 3.8
	Full MS	82.7 ± 3.3	93.5 ± 2.6
	Full MS + 3 g l^{-1} AC	45.4 ± 7.8	67.6 ± 7.8
	Full MS + 4.9 μ*M* IBA	74.8 ± 5.2	90.3 ± 4.2
T. pseudopectinatus	1/2 MS	85.5 ± 5.2	78.5 ± 3.6
	Full MS	91.4 ± 2.1	79.1 ± 3.8
	Full MS + 3 g l^{-1} AC	54.1 ± 3.6	74.6 ± 3.3
	Full MS + 4.9 μ*M* IBA	71.17 ± 2.6	74.0 ± 6.52
T. schmiedickeanus subsp. *flaviflorus*	1/2 MS	50.1 ± 8.3	81.0 ± 4.7
	Full MS	54.2 ± 5.9	93.1 ± 2.1
	Full MS + 3 g l^{-1} AC	53.5 ± 2.6	89.2 ± 8.9
	Full MS + 4.9 μ*M* IBA	57.4 ± 9.9	86.1 ± 1.9
T. schmiedickeanus subsp. *klinkerianus*	1/2 MS	77.9 ± 5.1	92 .0 ± 3.2
	Full MS	91.3 ± 5.1	89.1 ± 4.4
	Full MS + 3 g l^{-1} AC	60.9 ± 3.8	96.3 ± 7.2
	Full MS + 4.9 μ*M* IBA	73.6 ± 5.2	88.3 ± 3.3
T. schmiedickeanus subsp. *schmiedickeanus*	1/2 MS	89.1 ± 1.5	87.9 ± 4.0
	Full MS	92.0 ± 2.5	76.1 ± 3.9
	Full MS + 3 g l^{-1} AC	67.5 ± 2.5	98.3 ± 4.2
	Full MS + 4.9 μ*M* IBA	88.6 ± 2.6	96.3 ± 3.2
T. subterraneus	1/2 MS	90.3 ± 6.2	88.3 ± 6.3
	Full MS	94.2 ± 3.9	90.2 ± 4.3
	Full MS + 3 g l^{-1} AC	79.8 ± 4.6	94.3 ± 3.5
	Full MS + 4.9 μ*M* IBA	87.3 ± 7.4	89.5 ± 3.9
T. valdezianus	1/2 MS	70.0 ± 7.1	79.3 ± 8.7
	Full MS	84.4 ± 5.8	83.9 ± 6.1
	Full MS + 3 g l^{-1} AC	69.2 ± 3.0	90.3 ± 6.2
	Full MS + 4.9 μ*M* IBA	61.4 ± 4.3	81.3 ± 5.4

Ac, activated charcoal; 1/2MS, half-strength MS basal medium; Full MS, full-strength MS basal medium.

The production of roots was evaluated 6 wk after initiating the experiment. Values represent rooting frequencies of 50 shoots per treatment. Data were pooled from two independent experiments.

Survival percentages were determined 16 wk after transplantation.

75–90 d of incubation. However, these shoots were handled separately and were not included in the results due to the risk of somaclonal variation from having originated from callus.

In most cases, generated shoots were collected to be used in rooting experiments. However, when masses of shoots obtained through a first proliferation cycle were subcultured intact into fresh medium for a second proliferation cycle, an abundant secondary shoot proliferation appeared. This has already been reported for other cacti (Pérez-Molphe-Balch and Dávila-Figueroa, 2002) and it consists of the emergence of secondary shoots from the areoles of primary shoots. By this means it was possible to generate between 120 and 360 shoots in one culture vessel (Fig. 3*e*, *f*), which proves useful for the establishment of a continuous plant production system.

Shoot rooting took place in all treatments tested (Table 1; Fig. 3*g*, *h*), although the best response occurred in basal medium without activated carbon or IBA. Rooting frequencies ranged from 54.2% in *T. schmiedickeanus* subsp. *flaviflorus* to 94.2% in *T. subterraneus*. Mata-Rosas et al. (2001) report a rooting efficiency of 94–100% in *T. laui*. An interesting phenomenon observed at the rooting stage was *in vitro* flowering of several of the obtained plants (Fig. 3*i*). This is not surprising as it is known that *Turbinicarpus* has the capacity to produce flowers and fruit in juvenile stages (Anderson, 2001). These observations confirm this capacity also is manifested *in vitro*. Regarding adaptation to soil of rooted shoots, the survival rates were from 79.1% in *T. pseudopectinatus* (Fig. 3*j*) up to 98.3% in *T. schmiedickeanus* subsp. *schmiedickeanus*.

In conclusion, the *in vitro* proliferation systems developed for eight species and subspecies of the genus *Turbinicarpus* can be used in plant production destined for sustainable use, thus diminishing the existing pressures on wild populations. In the future, once adequate studies on genetic variability of the materials to be propagated are available, *in vitro*-generated plants could be reintroduced to their natural habitat.

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